EFFECT OF FERTILIZER AND SUCCESSIVE CUTTING ON GROWTH, YIELD, PHYTOCHEMICALS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF BERMUDA GRASS (*Cynodon dactylon*)

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries and references, which have been fully acknowledged.

10 October 2017

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ABSTRACT

Cynodon dactylon (L.) Pers. or commonly known as a Bermuda grass has been reported to have an antioxidant and antimicrobial properties. A series of experiments were conducted to examine the effect of fertilizers (organic A and B; inorganic NPK 15-15-15), fertilizer rates (12.5 or 25 kg N ha^{-1} per month) and successive cutting (first, second and third) on concentration of phytochemicals (total phenolic, total flavonoid, total saponin and total alkaloid contents) and antioxidant and antimicrobial activities of Bermuda grass. The organic fertilizer A was NPK 8-8-8, trace element, organic matter and humic acid meanwhile, organic fertilizer B was NPK 5-5-5, trace element, organic matter and effective microorganisms. First, second and third successive cutting was carried out repetitively on the same grass at 10, 15 and 20 weeks after planting, respectively. Growth parameter measurements were leaf width (mm), internodes length (cm), shoot density (tillers ha^{-1}), and clipping yield (kg of dry weight ha^{-1}). The results indicated that 25 kg N ha^{-1} per month of inorganic fertilizer increased grass shoot density significantly. Clipping yield was the highest at first successive cutting and 25 kg N ha^{-1} fertilizer application per month. The growth assessment supported the rationale for frequent mowing in turf grass management that it's improves new tiller production and reduces grass internode length and leaf width. For the phytochemical analysis, the addition of organic fertilizer B at 12.5 kg N ha^{-1} per month enhanced total flavonoid and antioxidant activity evaluated as lower IC 50 value and higher ferric reducing ability. There was a positive correlation between total phenolic and flavonoid content with antioxidant activity of the grass extract indicating medicinal value of the grass. With reference to harvest time, successive cutting was found to have reduced significantly the grass clipping yield. However, at either 12.5 or 25 kg N ha^{-1} application per month of inorganic fertilizer successive cutting was found to have increased the antimicrobial activity and phytochemical concentration of the grass. Successive cutting also found beneficial to improve antioxidant activity as it increased in number. In respect to antimicrobial activity, the grass extract showed a broad spectrum activity on the tested pathogens. Gram-positive rather than gram-negative bacteria were found to be susceptible to the antimicrobial activity of the grass extract. The MIC values were ranging 6.25 to 25 mg ml^{-1} for *Bacillus cereus* while 12.5 to 50 mg ml^{-1} for *Staphylococcus aureus* and *Salmonella typhi*. The MBC value was < 200 mg ml^{-1} for *B. cereus*, 50 to 200 mg ml^{-1} for *S. aureus* and 100 to 200 mg ml^{-1} for *S. typhi*. A positive correlation between total saponin content and antimicrobial activity indicated that saponin could be the main antimicrobial agent in the grass.

ABSTRAK

KESAN RAWATAN BAJA DAN PENUAIAN BERTERUSAN PADA PERTUMBUHAN, HASIL, FITOKIMIA, AKTIVITI ANTIOKSIDA DAN ANTIBAKTERIA BERMUDA GRASS (C. dactylon)

Cynodon dactylon (L.) Pers. atau dikenali sebagai rumput Bermuda telah dilaporkan mempunyai ciri aktiviti antioksida dan antibakteria. Tetapi maklumat mengenainya di Sabah dan Malaysia adalah terhad. Kajian ini dilaksanakan adalah untuk meneroka kesan jenis baja (organik A dan B; baja kimia NPK 15-15-15), kadar baja (12.5 atau 25 kg N ha^{-1} sebulan) dan penuaian berterusan (pertama, Kedua dan ketiga) terhadap jumlah kandungan fitokimia (phenolic, flavonoid, saponin dan alkaloid) dan aktiviti antioksida serta antimikrob ekstrak rumput Bermuda. Baja organik A mengandungi NPK 8-8-8, unsur lain tambahan, bahan organic dan asid humic manakala, baja organik B mengandungi NPK 5-5-5, unsur lain tambahan, bahan organic dan efektif mikrob. Penuaian secara berterusan melibatkan penuaian secara berulang terhadap rumput yang sama pada tiga jadual penuaian yang berbeza iaitu 10, 15 dan 20 minggu selepas penanaman telah dilaksanakan. Parameter pertumbuhan seperti lebar daun (mm), panjang ruas (cm), kepadatan (tillers ha^{-1}), dan hasil tuaian (kg of dry weight ha^{-1}) telah direkordkan. Penilaian terhadap pertumbuhan rumput menyokong rasional rutin pemotongan rumput turf secara kerap dalam rutin pengurusan rumput turf yang mana lebar daun mengecil, ruas menjadi pendek dan kepadatan rumput meningkat. Dari segi analisis kandungan fitokimia, didapati penggunaan baja organik B pada kadar 12.5 kg N ha^{-1}sebulan telah meningkatkan jumlah kandungan flavonoid dan kadar aktiviti antioksida rumput Bermuda yang dinilai melalui nilai IC 50 yang rendah dan keupayaan mengurai ferik yang tinggi. Korelasi positif di antara jumlah phenolic dan flavonoid terhadap aktiviti antioksida menunjukan kepentingan nilai perubatan rumput Bermuda. Skema penuaian berterusan didapati menyebabkan hasil tuaian menurun. Namun demikian, skema penuaian berterusan dan kombinasi aplikasi baja kimia pada kadar 12.5 atau 25 kg N ha^{-1} sebulan meningkatkan kandungan fitokimia dan aktiviti antimikrob. Penuaian secara berterusan juga turut meningkatkan aktiviti antioksida rumput. Dapatan kajian ini turut menunjukkan spektrum aktiviti antimikrob yang luas. Bakteria gram-positif didapati lebih terdedah kepada kesan antimikrob rumput berbanding bakteria gram-negatif. Kadar Perencatan Minimum (MIC) bagi Bacillus cereus ialah 6.25 - 25 mg ml^{-1}. Manakala, 12.5 - 50 mg ml^{-1} bagi Staphylococcus aureus dan Salmonella typhi. Kepekatan Bakterisidal Minimum (MBC) bagi B. cereus adalah didapati melebihi 200 mg ml^{-1} manakala 50 - 200 mg ml^{-1} bagi S. aureus dan 100 - 200 mg ml^{-1} bagi S. typhi. Korelasi positif di antara jumlah kandungan saponin dan aktiviti antimikrob menunjukan kebarangkalian bahawa saponin merupakan penyumbang utama aktiviti antimikrob ekstrak C. dactylon.

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LIST OF SYMBOLS

≤	Less than
≥	More than
µl	Microliter
°	Degree
®	Registered trade-mark
%	Percentage

LIST OF ABBREVIATIONS

AE	Atropine equivalent
DE	Diosgenin equivalent
FAO	Food and Agriculture Organization of the United Nations
GAE	Gallic acid equivalent
ha	Hectare
kg	Kilogram
MHA	Muller-Hinton Agar
MHB	Muller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
N	Nitrogen
QE	Quercetin equivalent
SPSS	Statistical Package for Social Science

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CHAPTER 1

INTRODUCTION

1.1 Background

Cynodon dactylon or known as Doob, Durva or Ghas in Ayurveda literature are among important medicinal plant documented in the traditional medicine system (Kumar *et al.*, 2011). This grass was used traditionally as a folk medicine to treat cough, cramps, dropsy, epilepsy, headache, wounds hypertension, asthma, measles, cancer, tumors and others (Nagori and Solanki, 2011). Previous findings stated that *C. dactylon* possess various type of secondary metabolite constituent and pharmacological activity such as antioxidant, antimicrobial, wound healing, antidiabetic, anti-cancer, anti-malaria and anti-chikungunya (Harisaranraj, Saravana Babu and Suresh, 2010; Khilifi, Hayouni, Cazaux, Moukarzel, Hamdi and Bouajila, 2013; Chandel and Kumar, 2015; Madhankumar, Jebakani, and Sundarapandian, 2015; Murali, Sivasubramanian, Vincent, Bala, Giridaran, Dinesh, Gunasekaran, Krishnasamy and Sathishkumar, 2015). It was acknowledged as a grass with great medicinal potential (Ashokkumar, Selvaraj, and Muthukrishnan, 2013). These findings proven that *C. dactylon* has a high potential to be developed to various type of herbal product or medicine.

In recent years, many explorative studies focusing on the benefit of various medicinal plants were conducted due to the raise in public concern on the side effect of synthetically synthesis drugs and the emergence of antibiotic resistance microorganism. The outbreak of antibiotic resistance pathogen was alarming; it was a threat not only to a nation but to the world as well. It was described that lack of new drugs development because of incentives privation and challenging regulatory are among main causal of this threat occurs apart from the overuse and misused of antibiotic (Ventola, 2015). The information point out the importance of new drugs development so that various types of drugs are accessible to prevent the pathogen resistance to single type of drug.

Formerly, since ancient times, plants have been intensively used as sources of medicine for various illnesses but none was used as antimicrobial agent (Cowan, 1999). Most of useful antibiotic was derived from the bacterial or fungal but not from the plant. For example, penicillin was the first natural antibiotic discovered from the fungus of *Penicillium* genus. However, eventually most antibiotic that generally derived naturally or synthetically from the fungal and bacteria was reported may have limited life span to be effective towards selective pathogen (Cowan, 1999), the emergence of bacteria antibiotic resistance in these days verify the statement.

As the result of this distressing situation, most researchers and pharmaceutical industries have been intensively researching for the alternative antimicrobial agents' source. Plants have been selected as the main subject for the alternative source of antimicrobial agent due to its beneficial effect on human health. The effort was worthwhile as it was found that various types of plants extract show promising antimicrobial properties toward various ranges of bacteria. The results deliver scientific evidence on the significance of medicinal plant documented in ancient literature. The scientific evidence urge more interest on the medicinal plant, creates opportunities in herbal industry as well as drawback and issue to be deal with.

There are various issues to be deal with medicinal plant industry. Inconsistent supply, poor traceability and questionable quality and purity of medicinal plants are among issue related with herbal product development (Kleter and Marvin, 2009). But the most concerning issue is the overharvesting and degradation of medicinal plant in the ecosystem (FAO, 2002). Medicinal plant was used to be collected from the nature, rapid harvesting may deplete the plant source. Overharvesting of medicinal plants from the wild can be mitigated in several ways.

Conservation plan would be useful, but a mere conservation without generating any revenue is not sustainable. Hence, the plants have to be still utilized or harvested for commercial purposes while conserve it. This entire problem was associated with the wild harvesting of medicinal plants. Therefore, cultivation of medicinal plant would be applicable approach to overcome the issue related to wild harvesting. However, little is known about the effect of medicinal plant cultivation on its medicinal value and production.

Cultivation of medicinal plant may involve several important modules such as selection of place (geography and environment), fertilization scheme, light exposure, watering schedule, harvesting schedule and others. Abiotic and biotic factors such as light intensity, water stress, nutrient supply and herbivore attack was reported could influence the quality and quantity of important secondary metabolite found in medicinal plants (Lambers, Chapin, and Thijis, 2008). 'Dao Di' terms was used by Chinese people in China to describe the premier quality of herbs cultivated or collected from the best area at ideal time (Huang and Gao, 2013), indicated it was crucial to determine the best cultivation area or soil fertilization scheme as well as harvesting technique and schedule for valuable medicinal plant.

Agronomic practices such as fertilization and harvesting are among crucial practices in medicinal plant cultivation. Fertilization provides macronutrient and micronutrient for plant growth and development, an optimum fertilizer application exhibit positive effect to plant quality and productivity thus, guarantee the growth and plant productivity maintained in a great state (Morris, 2007; White and Brown, 2010). According to Turgeon (2008), nitrogen is the most limiting nutrient in grass species, deficiency in the macronutrient and micronutrient may retard the plant growth and its productivity. Effect of fertilization on growth and productivity of medicinal plant was well documented (Wahba, Motawe and Ibrahim, 2014; Singh, Khan, and Naeem, 2016). Yet, information on effect of fertilization towards secondary metabolite concentration and its medicinal value is still lacking. Fertilization was reported able to alter the phytochemical and bioactive constituent capacity in plant (Lambers *et al.*, 2008). A positive and negative findings regarding the effect of fertilization on phytochemical and pharmacological activity of medicinal plants have been demonstrated in several studies (Okwu and Ukanwa, 2007; Hassan, Mijin, Yusoff, Ding and Wahab 2012; Hassan *et al.,* 2012; Naguib, El-Baz, Salama, Abd El Baky Hanaa, Ali and Gaafar, 2012; Ibrahim, Jaafar, Karimi, and Ghasemzadeh, 2013). These findings indicated the optimization in fertilization scheme during medicinal plant cultivation can enhance its productivity and quality, especially in term of increasing the concentration of targeted phytochemical for sequentially extraction and product development.

Meanwhile, successive harvesting is necessary to obtained raw material for extraction and product development. Most plant have a special ability to regenerate after being harvested or cut, and the ability was dependable on the plant physiology and severity of the defoliation itself (Dell, Hopkins, and Lamont, 1986). Shrubs and woody plant regrow a new shoots from the lateral bud in response to dormancy break when the terminal bud was cut or injured (Buchner,2012), meanwhile grass regrow a news shoots from the axillary bud or a completely new individual shoot called tiller emerges from the stolon or rhizome (Turgeon, 2008). This special ability of plant helps to assure the constant supply of medicinal plant, as the harvesting process can be conducted many times. Moreover, the act of cutting or removing the top growth of plant such as pruning, mowing and plucking helps improving new shoots growth, result in vigorous and lush shoot growth (Reich, 2010), thus, providing more shoots for next harvest cycle. However, there is a little knowledge on the effect of successive cutting or frequent harvest on growth, yield, phytochemical content, antioxidant and antimicrobial activities exhibited by *C. dactylon* species.

Yet, it was known that frequent cutting or harvesting has a quite similar effect with herbivore attack or feeding which is exposed the grass to wounding stress. Wounding stress was described influenced the plant growth, chemical composition and regulation (Łukaszuk and Ciereszko, 2012). Wounding stress was reported may induce the phenylpropanoid metabolism (Dixon and Paiva, 1995). Recent findings also proven wounding in plant able to trigger the signaling molecule and synthesis enzyme involved in phenolic metabolism such as phenylalanine ammonia-lyase (PAL), leads to enhancement in phenolic compound and other secondary metabolites production (Saltveit, 2000; Jacobo-Velázquez, González-Agüero, and Cisneros-Zevallos, 2015). The discoveries indicate that wound stress on plant can improved the production of secondary metabolites especially phenolic compounds. But, information on influence of consecutive cutting or harvest towards secondary metabolite production and pharmacological activity of *C. dactylon* is still lacking.

Plant secondary metabolites or also known as phytochemical are substances that does not have direct influence in important plant process such as photosynthesis and respiration (Taiz and Zieger, 2002), yet it was beneficial in ecological functions such as symbiotic interaction, allelopathy and plant defense (Lambers *et al.*, 2008). Although, secondary metabolite or phytochemical thought to be functionless in important plant process. But, currently it was considered as important substances in pharmaceutical and herbal industries. Various types of phytochemicals were reported beneficial in chronic disease prevention and treatments (Shahidi *et al.*, 2015; Wojtunik-Kulesza Oniszczuk, Oniszczuk and Waksmundzka-hajnos, 2016; Kaur *et al.*, 2016). For instance, flavonoid was able to inhibit nucleic acid synthesis, cytoplasmic membrane function and energy metabolism of bacteria resulted in inhibition of bacteria growth (Cushnie and Lamb, 2005). Furthermore, antioxidant activity exhibited by medicinal plant was described correlated to the amount of phenolic and flavonoid found in the plant itself (Ibrahim *et al.*, 2013). These findings verified secondary metabolite is a significant determinant influencing the pharmacological activity of medicinal plants. Therefore, it is necessary to ensure the production of this valuable compound would be enhances through the cultivation process yet, maintaining a good growth and productivity of the medicinal plant also should be taken into consideration to ensure the constant supplied of raw material for product development.

Hence, it was hoped this explorative study would give some basic information about the influence of fertilization and successive cutting towards growth, yield, the total phenolic, total flavonoid, total saponin and total alkaloid contents of *C. dactylon* in Sabah as well as its antioxidant and antimicrobial activities.

1.2 Justification of Study

Good agronomic practices are among important key to produce good quality and productive crops. Alike in medicinal plant cultivation, good agronomic practices may assist in high yielding production as well as high concentration of target compounds such as phenolic, flavonoid, saponin and alkaloid. Fertilization scheme is among crucial inputs in cultivation of crops as well as in medicinal plants. Recently, it was described fertilization influence the secondary metabolite content in medicinal plants. Harvesting is another necessary agronomic practice involved in medicinal plant cultivation, it ensure rapid and consistent supply of raw material for product development. Rapid harvesting has similar effect like herbivore attack which is exposed injury stress to plants and it was reported have negative and positive effect on plant growth and the production of important compound in plants. However, there is still lacking information on effect of consecutive harvesting as well as fertilization scheme on medicinal plant in Sabah and Malaysia especially on *C. dactylon.* Therefore, this study will provide basic information on the effects of different fertilizer treatments and successive cutting towards *C. dactylon*. The information would be useful in the future, if the grass is to be cultivated and harvested sequentially for secondary metabolites extraction and product developments.

1.3 Objectives

The objectives were as follow:

1. To evaluate the effect of different fertilizer scheme (types and rates) and successive cutting on growth and yield of *C. dactylon*.

2. To compare the influence of different fertilizer scheme (types and rates) and successive cutting on phytochemicals content and antioxidant activity of *C. dactylon*.